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Methanogen communities in stools of humans of different age and health status and co-occurrence with bacteria

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One sentence summary: *Methanomassiliicoccales* occur frequently and are temporarily persistent in stools of adult humans.

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ABSTRACT

Hydrogenotrophic methanogens live in a syntrophic relationship with the human gut microbiota as the terminal part of the anaerobic food chain. *Methanobrevibacter smithii* of the *Methanobacteriales* is the prevailing archaeal species. Recently, methylotrophic archaea of the novel order *Methanomassiliicoccales* were isolated from human stools. Few data exist on the prevalence, abundance, persistence and ecology of these methanogens in humans. This study investigated methanogen communities in 26 healthy and obese children (8–14 years) and 18 adults (28–78 years) using quantitative PCR. Samples were obtained from nine females before and after giving birth. Bacterial groups linked to the abundance of methanogens in adult females were identified using a 16S rRNA gene amplicon data set. A total of 89% and 65% of adults and children, respectively, carried *Methanobacteriales*. *Methanomassiliicoccales* were recovered from 50% of the adults and one child. Mean relative abundance of *Methanomassiliicoccales* in adults was lower than that of *Methanobacteriales* (0.10% versus 0.52%). Both *Methanobacteriales* and *Methanomassiliicoccales* formed stable communities in females before and after giving birth. *Methanobacteriales* co-occurred with bacterial taxonomic groups associated with the trophic chain from carbohydrate degradation to hydrogen and formate formation. Relative abundance was inversely correlated to *Blautia*. Negative correlation with little characterized groups within the *Clostridiales* indicated possible interactions of *Methanomassiliicoccales* with the bacterial community.

Keywords: *Methanomassiliicoccales*; *Methanobacteriales*; human stool

INTRODUCTION

The intestine of mammals harbours large and diverse communities of microbes that break down otherwise indigestible dietary fibres and proteins. Syntrophic interactions of intestinal microbes during fibre and protein degradation lead to the formation of short chain fatty acids (SCFAs; such as acetate, butyrate and propionate), organic acids (lactate and formate) and gases (hydrogen and carbon dioxide). The accumulation of hydrogen prevents the regeneration of the cofactors NADH⁺ and FADH⁺,

reducing the yield of ATP and SCFA formation; thus, an efficient removal of H₂ is essential to maintain the intestinal fermentation balance (Gibson, MacFarlane and Cummings 1993).

In the human gut, methanogens, acetogens and sulphate-reducing bacteria (SRB) are able to consume H₂. Acetogenesis is a commonly occurring metabolic process in the distal gut (Rey et al. 2010) that has been shown to be inversely correlated with methanogenesis (Doré et al. 1995). SRB can use H₂ as an electron acceptor to produce H₂S, thus competing with

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hydrogenotrophic methanogens, but can also grow syntrophically with some methanogens on lactate (Scholten, Culley and Brockman 2007).

Methanogens are strictly anaerobic archaea belonging to the Euryarchaeota, which today encompasses seven orders (Gaci et al. 2014). Most of the members of *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales*, *Methanocellales* and *Methanopyrales* perform methanogenesis from H_2/CO_2 . The versatile *Methanosarcinales* can produce methane from H_2/CO_2 , and/or acetoclastic and methylotrophic methanogenesis to different extents (Liu and Whitman 2008).

In the human gut, the hydrogenotrophic *Methanobrevibacter smithii* is the most abundant methanogen reducing CO_2 to methane with H_2 as the primary electron donor and also employing formate as substrate. In contrast, the less frequently detected *Methanosphaera stadtmanae* can only form methane from the reduction of methanol with H_2 (Liu and Whitman 2008; Scanlan, Shanahan and Marchesi 2008). In 2008, the existence of a novel order of putative methanogenic archaea closely related to the *Thermoplasmatales* in the human gut was proposed based on 16S rRNA gene and *mcrA* sequences (Mihajlovski, Alric and Brugère 2008). Physiological and genomic characterizations of isolates (*Methanomassiliicoccus luminyensis*) and enrichments ('*Candidatus* Methanomethylophilus alvus' and '*Candidatus* Methanomassiliicoccus intestinalis') derived from human stools have since verified that the *Methanomassiliicoccales* clade indeed contains methanogens, and that members perform H_2 -dependent methanogenesis from methanol and also utilize methylamines (Borrel et al. 2012; Dridi et al. 2012a; 2013a,b; Poulsen et al. 2013; Brugère et al. 2014). Additional data indicated that this novel order of methanogens inhabits similar habitats to *Mbb. smithii*. Beside the human gut, *Methanomassiliicoccales* have been detected in the oral cavity (Dridi, Raoult and Drancourt 2011; Horz, Seyfarth and Conrads 2012).

In herbivorous ruminants, the abundance and diversity of hydrogenotrophic and methylotrophic methanogens have been analysed in many species and dietary conditions (St-Pierre and Wright, 2013). Much fewer data exist on the methanogen communities in the human gut, especially for *Methanomassiliicoccales*. It was therefore the aim of this study to explore the stools of human subjects of different ages and health conditions for the prevalence, abundance and persistence of *Methanobacteriales* and *Methanomassiliicoccales* and to identify possible linkages of methanogen abundance with bacterial groups. Therefore, previously generated data sets were reanalysed with respect to the presence of metabolically different methanogens.

MATERIALS AND METHODS

Sampling

Samples were collected from 18 adults [5 elderly (all female, >72 years); 9 females, 28–40 years; and 4 males, 32–38 years] and from 26 children, 8–14 years old, in previous studies (Payne et al. 2011, 2012; Jost et al. 2014). From females N1, N4, N8, N12, N15, N16 and N18, samples were collected 3–7 weeks pre-partum (sampling day 1), and at days 3–6, 9–14 and 25–30 post-partum (sampling days 2, 3 and 4); from females N2 and N20, samples were obtained 3–7 weeks pre-partum, and at days 3–6 post-partum. Male N1 was sampled twice within 4 months. Children were of normal weight (NW, $n = 13$) or obese and overweight (OW, $n = 13$); the average body mass index (BMI) of overweight and obese children was 25.5 kg/m^2 (versus 16.8 kg/m^2 in normal weight children). From two girls (NWN1 and OWN1), samples

were obtained at two time points which were 1 year apart (Payne et al. 2011, 2012). DNA had been isolated using the FastDNA SPIN Kit for soil (MP Biomedicals, Switzerland) (Payne et al. 2011, 2012; Jost et al. 2014). Ethics approvals were obtained in the course of those studies.

Quantitative PCR

The abundance of methanogens was determined using quantitative PCR and primers 915F (5'-AGG AAT TGG CGG GGG AGC AC-3') and 1100AR (5'-TGG GTC TCG CTC GTT G-3') targeting *Methanobacteriales* (Tymensen and McAllister, 2012), AS1 (5'-CAG CAG TCG CGA AAA CTT C-3') and AS2 (5'-AAC AAC TTC TCT CCG GCA C-3') targeting *Methanomassiliicoccales* (Mihajlovski et al. 2010), and 519F (5'-CAG CCG CCG CGG TAA TAC C-3') and AS2 (see above) which were optimized for methylotrophic methanogens identified in the oral cavity (Horz, Seyfarth and Conrads 2012). Total bacteria were quantified using primers TBfor (5'-CGG YCC AGA CTC CTA CGG G-3') and TBrev (5'-TTA CCG CGG CTG CTG GCA C-3') (Lee, Zo and Kim 1996).

Reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Switzerland) and the Kapa SYBR FAST qPCR mastermix (Biolab Scientific Instruments SA, Switzerland). Thermal cycling consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles consisting of denaturation at 95°C for 3 s, annealing at 60°C for 10 s and elongation at 72°C for 25 s. Templates for standards for hydrogenotrophic and methylotrophic methanogens were amplified from faeces derived from minipigs. PCR amplicons were cloned into PGEMT Easy Vector and heterologously expressed in *Escherichia coli* according to the instructions of the supplier (Promega, Switzerland). Standard curves were prepared from 10-fold dilutions of linearized plasmids harbouring the 16S rRNA gene of interest. The linear detection range was between log 2.0 and log 8.0 gene copies per assay for total bacteria (Primer TBfor and TBrev, approximate threshold per gram of stool: log 4.6 gene copies/g), log 2.3 and log 8.3 for *Methanobacteriales* (915F and 1100R, approximate threshold per gram of stool: log 4.9 gene copies/g), and log 2.3 and log 9.3 for *Methanomassiliicoccales* using primers AS1 and AS2, or AS2 and 519F (approximate threshold per gram of stool: log 4.6 gene copies/g). To verify the specificity of amplification, melting curve analysis and agarose gel electrophoresis for amplicon size control were performed. The relative proportion of methanogenic archaea in the total faecal microbiota was calculated as the ratio of 16S rRNA gene copies of methanogenic archaea:16S rRNA gene copies of archaea and bacteria. The proportion of *Methanomassiliicoccales* in the methanogenic community was calculated as the ratio of 16S rRNA gene copies of *Methanomassiliicoccales* divided by the sum of 16S rRNA gene copies of *Methanobacteriales* and *Methanomassiliicoccales* methanogens.

Restriction fragment length polymorphism (RFLP)

From samples that were positive for *Methanomassiliicoccales* in qPCR, we attempted to amplify 16S rRNA genes using PCR and primer pair 519F and AS2. Due to low abundance, amplicons were only obtained from some samples (female N1, sampling days 2 and 3; female N2, sampling day 2; female N4, sampling days 2 and 3; female N12, sampling day 1; OWN1 sampling days 1 and 2; and one elderly E2).

Amplicons were cloned into PGEMT Easy Vector and heterologously expressed in *E. coli* according to the instructions of the supplier (Promega). Amplicons obtained from colony PCR of at

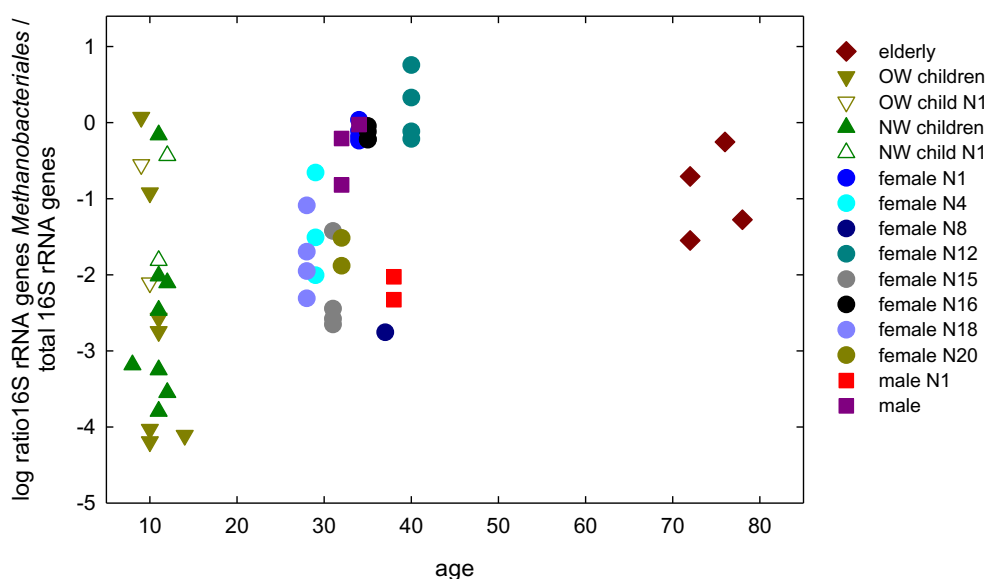


Figure 1. Log relative abundance of *Methanobacteriales* in faeces of humans of different ages (children, adults and the elderly) and health conditions (normal weight and obese, pregnant and after birth). Gene copies were determined using quantitative PCR. The relative proportion of hydrogenotrophic methanogens in the total faecal microbiota was calculated as the ratio of 16S rRNA gene copies of hydrogenotrophic methanogens to the sum of 16S rRNA gene copies of archaea and bacteria.

least eight colonies using primers SP6 and T7 were directly used for RFLP using the restriction enzyme *HpyCH4V* (New England Biolabs) and sequencing. To generate the RFLP pattern, amplicons were incubated with *HpyCH4V* for 4 h at 37°C followed by inactivation at 65°C for 20 min. RFLP patterns were separated on 3% agarose gels and visualized after staining with ethidium bromide. Representative clones were sequenced; sequence files are summarized in the Supplementary data file.

Phylogenetic analysis of 16S rRNA gene sequences obtained in this study

16S rRNA genes were aligned and cut using CLUSTALW implemented in BioEdit. Phylogenetic analysis was performed using MEGA5 and the maximum likelihood analysis applying the Jones–Taylor–Thornton substitution model and default settings. Bootstrap support was calculated for 100 replicates; *Sulfolobus islandicus* and *Nitrosocaldus yellowstonii* were applied as outgroups.

Reanalysis of 16S rRNA amplicon sequences of females

To identify bacterial groups that co-occurred with methanogens, we reanalysed 16S rRNA gene amplicon sequences generated from stools of seven females (N1, N4, N8, N12, N15, N16 and N18) before and after giving birth (Jost et al. 2014). This data set had been generated by unidirectional barcoded multiplex sequencing using a 454 Life Sciences GS FLX system in combination with Titanium chemistry and was deposited in NCBI's short read archive under accession number SRA065797. Primers used targeted the V6 hypervariable 16S rRNA region (Andersson, Lindberg and Jakobsson 2008). 16S rRNA gene sequences derived from this data set were compared with a recent version of the SILVA database (SilvaMod v106, provided by CREST) using BLAST and imported into MEGAN (Min Score = 150, Top Percent = 10, Min support 1) (Mittra, Stärk and Huson 2011; Lanzén et al. 2012). Statistical analysis was performed with SigmaPlot 11 using Spearman correlation analysis and unpaired t-test, a P-value <0.05 was considered significant. If normality test failed, the Mann–Whitney Rank Sum Test was applied.

RESULTS AND DISCUSSION

Frequency of detection and abundance of hydrogenotrophic methanogens

According to breath tests, between 30% and 60% of Western adults are positive for methanogens (Levitt et al. 2006). However, some studies have reported higher percentages of carriers (Dridi et al. 2012b; Jalanka-Tuovinen et al. 2011). *Methanobacteriales* were the most frequently detected and the most abundant methanogens (mean relative abundance 0.44%) in most samples from all age groups (Fig. 1; Supplementary Fig. S1). A total of 89% (16/18) of the elderly and adult subjects harboured *Methanobacteriales*, confirming the high occurrence reported by Dridi et al. (2012b). *Methanobacteriales* were detected in the stools of 65% of the children (17/26). The mean relative abundance of *Methanobacteriales* in children was lower than in adults (0.15% versus 0.52%) (Fig. 1) and there was a general increase of prevalence and relative abundance of *Methanobacteriales* with age (correlation coefficient 0.489, $P < 0.001$). Results from previous studies using non-cultivation-based methods are contradictory. Dridi et al. (2012b) observed a high prevalence (88%) of *Methanobacteriales* already at age 0–10 (99 donors). In contrast, Stewart, Chadwick and Murray (2006) reported a lower prevalence in general and an age-dependent increase in 12 adults and 40 children; and Mihajlovski et al. (2010) did not recover *Mbb. smithii* from newborns ($n = 23$) but from all adults and elderly tested ($n = 28$).

From seven females, a time series of four samples was obtained, one before and three after giving birth; females N2 and N20 donated only one sample post-partum. Here we observed two groups: four females (N1, N12, N16 and N20) possessed stable communities of *Methanobacteriales* with mean relative abundance of 0.71–2.30%, while the other four females (N4, N8, N15 and N18) harboured *Methanobacteriales* at a mean relative abundance of $\leq 0.07\%$ (Fig. 2A). *Methanobacteriales* were not recovered from female N2.

It was previously shown that *Methanobacteriales* form stable communities in their host (Jalanka-Tuovinen et al. 2011). The present study further indicated that archaeal communities

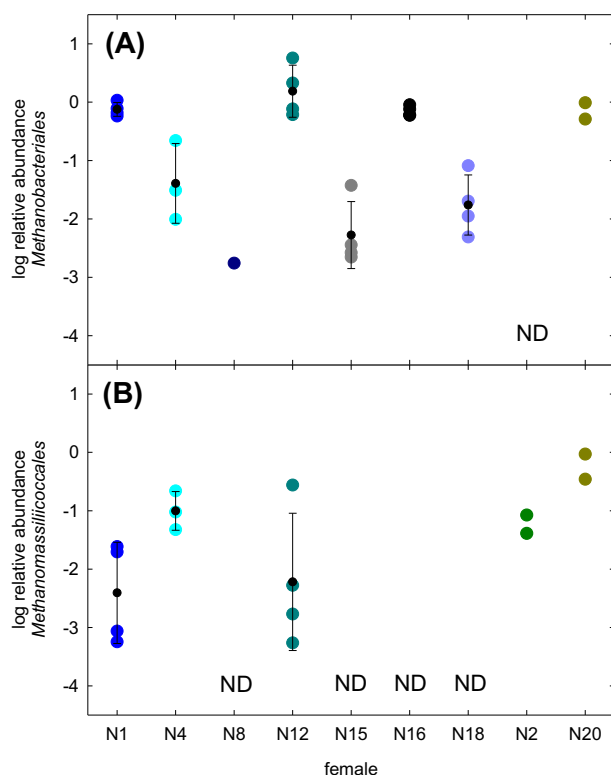


Figure 2. Temporal stability of *Methanobacteriales* (A) and *Methanomassiliicoccales* (B) in females before and after giving birth. In female N8, *Methanobacteriales* could only be detected at one time point; *Methanobacteriales* were not recovered from female N2. *Methanomassiliicoccales* were not detected in females N2, N8 and N18. ND, not detected.

remained persistent before and after giving birth, similar to what was reported for the faecal bacterial microbiota (Jost et al. 2014).

Hydrogenotrophic methanogens in obese and normal weight children

In stools of normal weight and obese children, the abundance of selected bacterial groups (*Bacteroides*, the butyrate-producing *Rosburia-Eubacterium rectale* group and *Faecalibacterium prausnitzii*, and sulphate-reducing bacteria) was determined using qPCR, and no variations related to weight could be observed (Payne et al. 2011). However, increased faecal levels of butyrate and propionate indicated increased metabolic activity in obese children (Payne et al. 2011). Despite the differences in metabolic activity, we did not observe any correlation between BMI and levels of hydrogenotrophic methanogens. Whether weight status of the host impacts the level of methanogens still remains unclear. Zhang et al. (2009) reported higher numbers of *Methanobacteriales* in obese humans together with enrichment of H_2 -producing *Prevotella*. According to another study investigating the occurrence of methanogens in twins, the abundance and prevalence of *Mbb. smithii* were not related to weight (Hansen et al. 2011), while a German study even observed reduced abundance of *Methanobrevibacter* in obese subjects (Schwiertz et al. 2009).

Prevalence and abundance of methylotrophic methanogens

Of the adults, 50% (9/18) possessed *Methanomassiliicoccales*. *Methanomassiliicoccales* were detected in three out of five of the

elderly (relative abundance 0.024–0.128%) and could be identified in five females and one male (M4, relative abundance 0.302%) (Supplementary Figs S1 and 2B). Only one overweight 9-year-old girl (BMI 21) harboured *Methanomassiliicoccales*. Here, we confirmed the age-dependent increase of prevalence of *Methanomassiliicoccales* from infancy to adulthood that was previously reported (Dridi et al. 2012b; Mihajlovski et al. 2010).

In ruminants and also wallabies, *Methanomassiliicoccales* constituted up to 90% of the *Euryarcheota* community (St-Pierre and Wright, 2013); the fraction of *Methanomassiliicoccales* in humans as determined in this study was substantially lower in most donors (Supplementary Fig. S1). Nevertheless, *Methanomassiliicoccales* outnumbered *Methanobacteriales* in child OWN1 and females N4 and N2 (Supplementary Fig. S1).

Few data exist on the temporal stability of *Methanomassiliicoccales* in the human intestine. In females N1, N4 and N12, *Methanomassiliicoccales* were repeatedly recovered (mean relative abundance 0.011 ± 0.012 , 0.120 ± 0.088 and $0.071 \pm 0.136\%$, respectively). *Methanomassiliicoccales* were also present in N2 and N20 at both sampling dates (Fig. 2B). Relative abundance was also consistent in the overweight child OWN1, with 0.135% and 0.518% relative abundance at sampling days 1 and 2, respectively.

RFLP separated clones in two patterns, which were detected in OWN1, FN1, FN4 and FN12. Only one pattern was observed in E1 and FN2. Phylogenetic analysis placed sequences within the gastrointestinal cluster of the *Methanomassiliicoccales* close to clone Mx-02 (Fig. 3; Borrel et al. 2013a; Mihajlovski et al. 2010). Gaci et al. (2014) suggested niche partitioning of different species of *Methanomassiliicoccales* similar to *Mbb. smithii* and *Methanobrevibacter oralis*. Our data point to high occurrence and stable persistence of at least the Mx-02 phylotype of the *Methanomassiliicoccales* in the human gut.

Co-occurrence of *Methanobacteriales* and *Methanomassiliicoccales* with intestinal bacteria

To identify co-occurrence of bacterial families and genera with methanogens, we reanalysed a 16S rRNA gene amplicon data set generated previously from females with four sampling dates before and after birth (N1, N4, N8, N12, N15, N16 and N18; Jost et al. 2014).

Four taxonomic groups (unclassified *Bifidobacteriaceae*, unclassified *Veillonellaceae*, *Dialister* and unclassified *Ruminococcaceae*) and a single environmental lineage within the *Tenericutes* (RF9) were significantly more prevalent in females N1, N12 and N16, with a mean *Methanobacteriales* community of $>0.71\%$ (Fig. 4), while the mean relative abundance of unclassified *Lachnospiraceae* and the genus *Blautia* were significantly higher in the group of females (N4, N8, N15 and N18) with mean relative abundance of *Methanobacteriales* $<0.07\%$.

Lachnospiraceae and *Ruminococcaceae* are functionally diverse families belonging to the order *Clostridiales*. Abundance changes of phylotypes of these families occurred in dysbiosis; several *Clostridiales* phylotypes have been positively correlated with *Mbb. smithii*, indicating intragroup variations in response to environmental changes or the presence of methanogens (Berry et al. 2012; Hansen et al. 2011). Here, unclassified *Ruminococcaceae* co-occurred with *Methanobacteriales* in accordance with previous observations (Fig. 4; Hoffmann et al. 2013). The syntrophic relationship of *Ruminococcus* and archaea enables the bacteria to produce twice as much ATP if hydrogen is consumed by the archaeon (Stams and Plugge 2009). In contrast, the relative abundance of unclassified *Lachnospiraceae* and *Blautia* was negatively

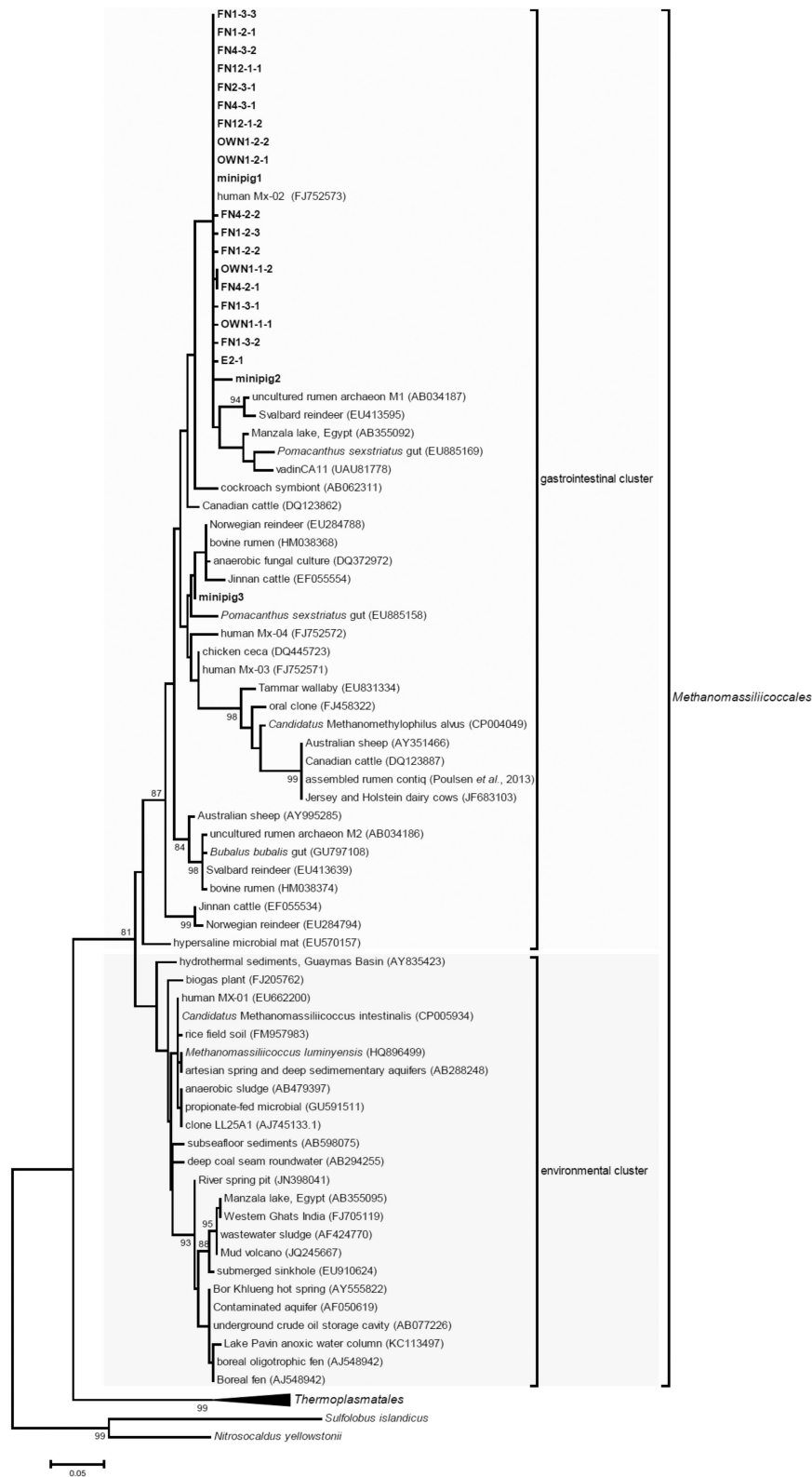


Figure 3. Environmental distribution of the clones obtained in this study. Sequences were phylogenetically analysed from partial sequences of the 16S rRNA gene (320 bp) using maximum likelihood analysis. Clones derived in this study are printed in bold. Bootstrap values below 80 are not shown. OWN1(donor)-2(sampling day)-1(clone number).

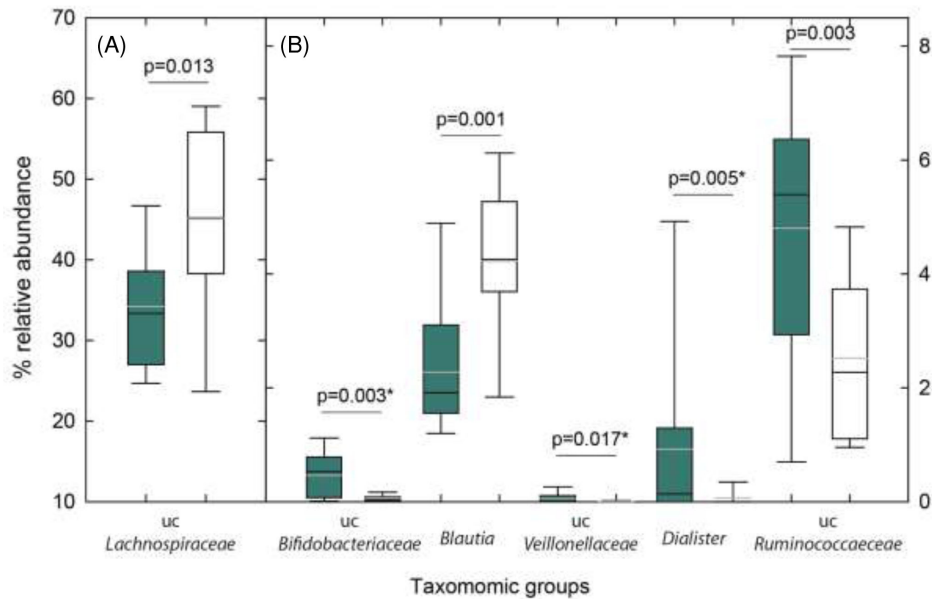


Figure 4. Box plots of relative abundance of taxonomic groups in mothers carrying *Methanobacteriales* at mean relative abundance >0.71% (blue fill) or <0.07% (white fill). Borders of the box indicate the 25th and 75th percentiles, and whiskers indicate the 10th and 90th percentiles. Median and mean are drawn in black and grey, respectively. Outliers are not shown. The mean relative abundance of bacterial taxonomic groups was compared using t-test. If the normality test failed, Mann–Whitney Rank Sum test was employed (indicated with *). uc, unclassified.

correlated to the abundance of *Methanobacteriales* (Table 1). *Blautia* encompasses species that can produce acetate from hydrogen and carbon dioxide (Bernalier et al. 1996) and thus compete with hydrogenotrophic methanogens for substrate.

The abundance of *Dialister* and unclassified *Veillonellaceae* positively correlated with the abundance of *Methanobacteriales* (Table 1). *Veillonellaceae* utilize the metabolic end-products of carbohydrate-fermenting bacteria as energy and carbon sources metabolizing lactate into propionate and H_2 .

Interestingly, the mean relative abundance of unclassified *Bifidobacteriaceae* was significantly higher in females possessing *Methanobacteriales* at >0.71% (Fig. 4). *Bifidobacteriaceae* do not produce hydrogen but, for example, *Bifidobacterium bifidum* can cleave pyruvate to acetate and formate (Winter 1980). Mixed cultures of *B. bifidum* and *Mbb. smithii* produced methane from glucose (Winter 1980).

The relative abundance of *Methanomassiliicoccales* was negatively correlated with that of unclassified *Lactobacillales* and

Coriobacteriaceae, and with functionally uncharacterized groups of the *Clostridiales* (Table 1). Methyl-containing compounds (e.g. methanol and trimethylamine), serving as substrates for methylotrophic methanogens, can be derived from microbial degradation of pectin, betaine, choline and L-carnitine which are present in various foods (Bradbeer, 1965; Mitchell, Chappell and Knox 1979; Schink and Zeikus, 1980). Clostridia are capable of releasing trimethylamines from amine compounds (Möller, Hippe and Gottschalk 1986). However, whether there is a substrate-related link between *Methanomassiliicoccales* and the inversely correlating bacterial taxonomic groups remains to be elucidated.

In summary, *Methanobacteriales* occurred more frequently and were more abundant than *Methanomassiliicoccales* in most individuals. When present, *Methanomassiliicoccales* were temporarily persistent. Negative correlation of *Methanomassiliicoccales* with little characterized groups within the *Clostridiales* indicated possible interactions with the gut microbiota.

Table 1. Taxonomic groups linked to the presence of *Methanobacteriales* and *Methanomassiliicoccales*.

Methanobacteriales				Methanomassiliicoccales			
Taxonomic group	Bacterial order	Correlation coefficient	P-value	Taxonomic group	Bacterial order	Correlation coefficient	P-value
uc ^a Porphyromonadaceae	Bacteroidales	0.403	0.033	uc Lactobacillales	Lactobacillales	−0.736	0.008
uc Lachnospiraceae	Clostridiales	−0.413	0.029	uc Coriobacteriaceae	Coriobacteriales	−0.682	0.019
Blautia	Clostridiales	−0.503	0.007	uc Peptostreptococcaceae	Clostridiales	−0.647	0.029
uc Veillonellaceae	Selenomadales	0.497	0.007	Peptostreptococcaceae	Clostridiales	−0.669	0.003
Dialister	Selenomadales	0.535	0.004	Incertae Sedis			
				uc Ruminococcaceae	Clostridiales	−0.624	0.035
				Ruminococcaceae Incertae Sedis	Clostridiales	−0.843	<0.001
				uc Clostridiaceae	Clostridiales	−0.633	0.032
				Family XIII Incertae Sedis	Clostridiales	−0.670	0.020

^auc unclassified.

Future (longitudinal) studies investigating both the *Methanomassiliicoccales* communities in the oral cavity and stools, together with physiological and genomic studies of isolates and enrichments, will shed further light on the lifestyle of methylo-trophic methanogens in the human gastrointestinal tract.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

Conflict of interest. None declared.

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